SURVIVAL OF MICROBIAL SPORES UNDER SEVERAL TEMPERATURE AND HUMIDITY CONDITIONS

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INTRODUCTION

Experimental procedures for measuring the survival rates of bacterial spores as a function of relative humidity in sealed plastic containers at 22° and 45°C were described in the previous progress reports (December, 1968 and June, 1969). During the current reporting period a number of experiments previously initiated have been completed and additional experiments have been performed.

OBJECTIVE

The objective of this project is to determine the effect of relative humidity and relatively low temperatures (<90°C) on the long-term survival of bacterial spores on surfaces. It is possible that there may be temperature and humidity conditions acceptable to space-craft engineers which will produce a relatively rapid rate of reduction in the microbial spore population. The effect of the conditions during either space hardware assembly or post-assembly storage could be used to reduce the terminal sterilization bio-load.

EXPERIMENTAL PROCEDURES

The procedures for obtaining the desired relative humidity conditions in sealed plastic containers were previously described in Progress Report #1, December, 1968. Details of the silica gel preparation are included in Appendix A of this report.

Experiments which have been concluded during the report period can be summarized as follows:

1. Final data analyses have been completed for a 28-week experiment to determine survival rates of Bacillus subtilis

var. niger (spore code AAAA) at 22°C and 45°C at <10%, ∿50%, and >90% RH in the closed system and 22°C and 30% RH with the spores exposed to the airstream of a laminar downflow room. Data through twenty weeks were presented in the previous report.

2. A 12-week experiment using the same species of microorganisms at 45°C and <10%, ∿25%, ∿35%, and ∿50% has also been completed and analyzed. The main purpose of this experiment was to determine the effect of relative humidities intermediate between <10% and ∿50% because these humidities will be more acceptable to spacecraft engineers for storage of components than humidity of 50% or higher.
3. An 8-week experiment has been completed and analyzed, again using the same suspension of Bacillus subtilis var. niger at 60°C and <10%, ∿50%, and >90% RH.
4. A 12-week experiment has been completed and analyzed using a suspension of spores isolated from garden soil. The soil suspension was stored at 50°C for 48 hours and then suspended in ethanol to destroy the vegetative cells. Survival rates were determined at 45°C and <10%, ∿50%, and >90% RH.
5. Based on data from the above experiments, a definitive series of experiments has been initiated at 45°C. This series can be summarized as follows:

   The objective of these experiments is to evaluate the following elements in the procedure used to determine D-values of Bacillus subtilis var. niger spores stored at <1%, <2%, ∿30%, ∿50% and >90% RH at 45°C.

   a) Strip material; glass vs. stainless steel
   b) Suspending fluid of the spores; 95% ethanol vs. water
   c) Different spore crops; spores produced on Tam agar vs. spores produced in SSM-10 liquid medium
   d) Box-to-box variation

   Three experiments will be performed. The relative humidities will be <1%, <2%, ∿30%, ∿50%, and >90%. A summary of the experimental design is shown in Table 1.1.

Table I.1 - Summary of Experiments on the Survival of Bacillus subtilis var. Niger Spores Stored at 45°C - <1%, <2%, ∿30%, ∿50%, and >90% Relative Humidity

Relative Humidity	# of Strips	Spore Susp.*	Strip Mat.	# of Strips	Spore Susp.*	Strip Mat.	# of Strips	Spore Susp.	Strip Mat.
<1% Dry Air	16	AAAA	SS**	16	AAAA	SS	16	AAAA	SS
<2%	8	AAAA	SS	8	AAAA	SS	8	AAAA	SS
Silica Gel	8	AAAA	g***	8	AAAB	SS	8	AAND	SS
∿30%	16	AAAA	SS	16	AAAA	SS	16	AAAA	SS
∿50%	8	AAAA	SS	8	AAAA	SS	8	AAAA	SS
	8	AAAA	g	8	AAAB	SS	8	AAND	SS
>90%	8	AAAA	SS	8	AAAA	SS	8	AAAA	SS
	8	AAAA	g	8	AAAB	SS	8	AAND	SS

* See Appendix B for details of the spore code

** 1 x 2 inch stainless steel strips

*** 1 x 2 inch glass strips

To achieve the <1% relative humidity, pint jars that can be hermetically sealed will be used to contain the strips. Strips will be inoculated, equilibrated for 20-24 hours, placed in jars and sealed inside a glove box with an atmosphere of flowing dry air (relative humidity ≤1%). Jars, covers, strips, etc. will be dried over silica gel before being placed in the glove box. The moisture in the atmosphere of the glove box will be analyzed with a Bell and Howell (C.E.C.) moisture analyzer. From these data the relative humidity in the jar at 45°C will be calculated. There are no plans to check the humidity in the jars after they have been sealed. The other relative humidity conditions will be achieved as previously reported using silica gel and water.

Two containers at <1%, <2%, and ∿30% relative humidity will be analyzed every two weeks for a period of ten weeks. Strips held at ∿50% and >90% RH will be analyzed weekly for a period of five weeks. Where two strip materials or spore suspensions are being evaluated, four strips of each material will be placed in a container. Sterile teflon strips will be used to separate strips placed back to back.

Eight strips from the glove box and eight from the downflow room will be processed as zero-time controls.

To date only Experiment I has been carried out but the data analysis for this experiment has not yet been completed.

6. A supplementary experiment was also carried out to ascertain the effect of utilizing higher agar concentrations when plating greater quantities of diluent. This practice has been followed in all previous experiments in an effort to equalize the available nutrient per given volume of diluent. The experiment was carried out as follows:

   Approximately 10^5 spores were deposited on each of five 1" x 2" stainless steel strips and were allowed to dry. Each strip was placed in a 125 ml flask, 50 ml of buffer were added, and the flask was then insonated for two minutes.

   Dilutions of the eluate were then made with sterile buffer so that by plating 0.1, 1.0, 10, and 30 ml aliquots, the same number of organisms would be present per dilution. (See Figure 1.1, page 5)

   Twenty ml of single strength TSA were used for plating the .1 and 1.0 ml dilutions, fifteen ml of 1 1/2 strength TSA for the 10 ml dilutions, and thirty ml of double strength TSA for the 30 ml dilutions.

7. A second supplementary experiment was carried out to determine whether two different pipettes would deliver equal quantities (.02 ml) of spore suspension. We also wanted to find out whether there was a trend toward either increasing or decreasing deposit concentrations with time between mixing sequences for the spore suspension. The experiment was carried out as follows:

   Thirty-two 1" x 2" stainless steel strips were contaminated with .02 ml of an alcohol suspension of Bacillus subtilis var. niger spores, sixteen using pipette A (downflow room) and sixteen using pipette B (crossflow room). A vortex mixer was used on the spore suspension after inoculating each of the eight strips with each pipette. Immediately after drying, the strips were transferred to sterile 125 ml flasks and processed alternately.

Figure I.I - Graphic Experimental Design Studies to Determine the Effect of Utilizing Higher Agar Concentrations when Plating Larger Quantities of Diluent.

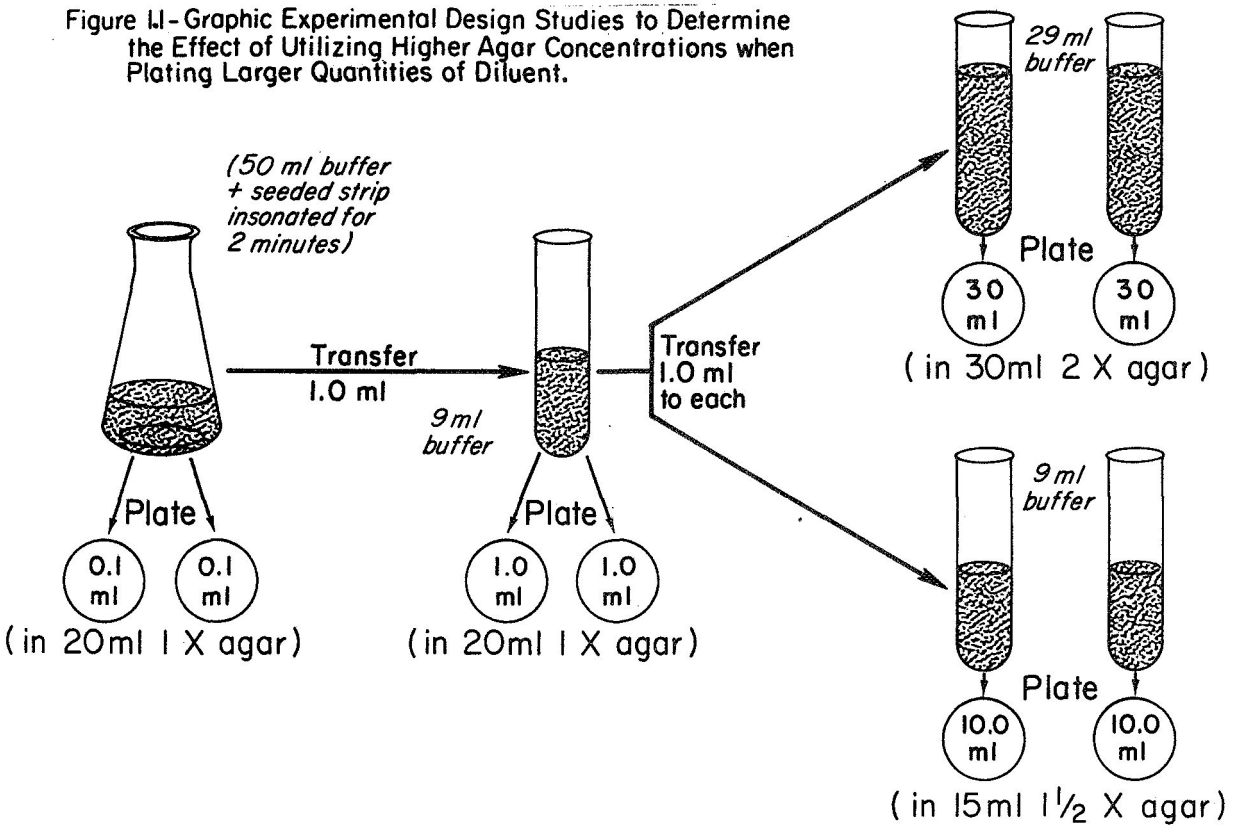

Figure I.1 - Graphical experimental design scheme for studies to determine the effect of utilizing higher agar concentrations when plating larger quantities of diluent

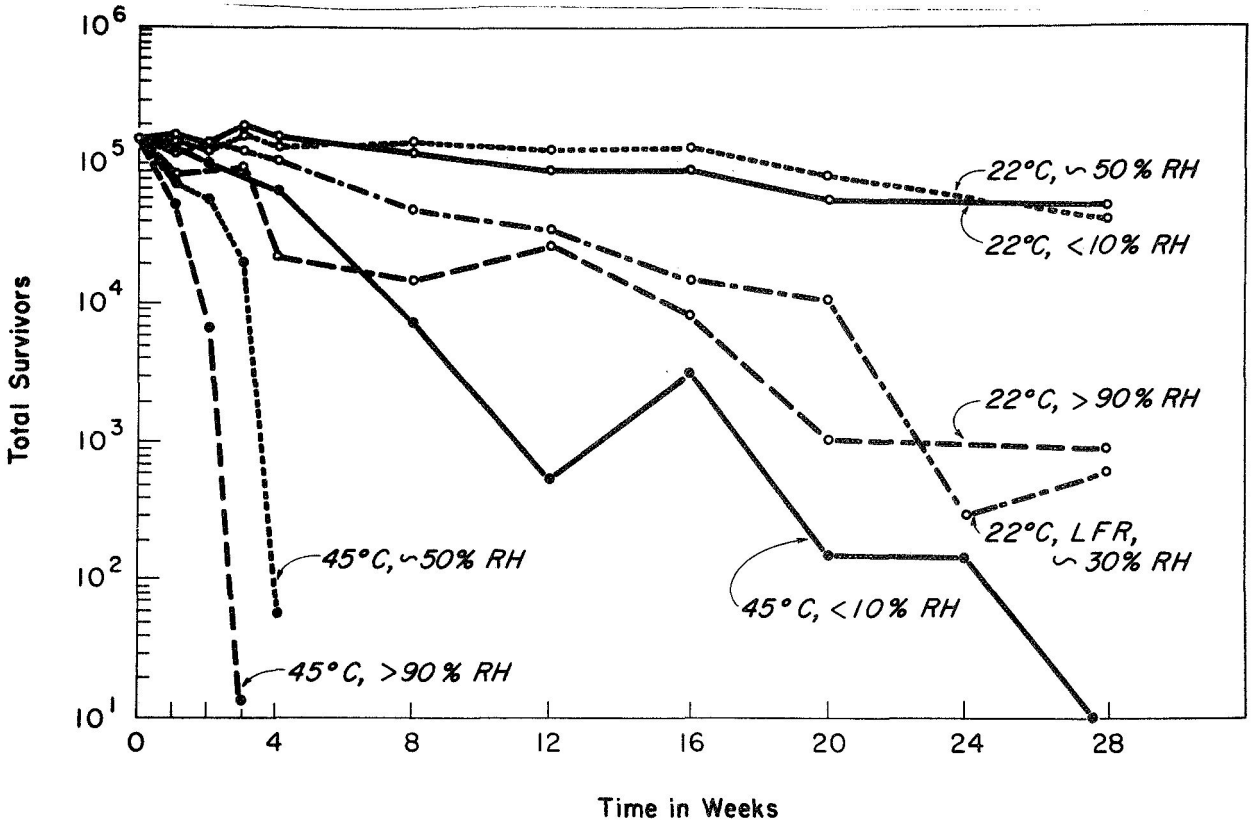

Figure I.2 - Survival of Bacillus subtilis var. niger spores at 22 and 45°C as a function of relative humidity during a 28-week experiment period

RESULTS

Figure I.2 represents the survival of the Bacillus subtilis var. niger spores at 22°C and 45°C during the 28-week experiment. Figure I.3 (see page 8) illustrates the survival at 45°C at the intermediate relative humidities during the 12-week experiment, while Figure I.4 (see page 8) depicts the same information at 60°C during the 8-week experiment at that temperature. The data from all of these experiments are summarized in Table I.2 and included are the range of relative humidities recorded and the D-values calculated from the slope of the least squares regression lines of log N vs. time.

Table I.2 - D-values of Bacillus subtilis var. niger Spores as a Function of Relative Humidity and Temperatures of 22°, 45°, and 60°C

Temperature °C	% RH Range	Length of Experiment (weeks)	N_o (x 10^5)	D-value (days)
22	<2	28	1.5	391.5
	30-40 (LFR)	28	1.5	74.3
	50-59	28	1.5	540.1
	88-96	28	1.5	89.1
45	<2-9	28	1.5	47.1
	<2-8	12	2.6	26.5
	24-27	12	2.6	18.9
	35-38	12	2.6	15.2
	55-57	28	1.5	7.1
	53-55	12	2.6	7.6
	96-100	28	1.5	4.4
60	<2-8	8	1.7	11.4
	53-55	8	1.7	2.7
	94-95	8	2.1	2.2

The relationship between D-value and relative humidity at each temperature tested is illustrated in Figure 1.5 (see page 8). It is apparent that in the ranges tested both the temperature and humidity significantly affected spore survival. The most rapid die-away occurred at the higher temperatures and at the higher humidities. Relatively little die-away was observed at 32°C, particularly in the closed systems and where the humidity was below 60%. In tests where the spores on strips were exposed to the laminar flow air stream the die-away was more rapid than it was in the very high humidity condition in the closed containers. However, even these conditions at 22°C resulted in considerably longer D-values than those in the lowest humidity in experiments at 45°C. The D-values of less than twenty days observed at 45°C, even at relative humidity as low as ∿25%, indicate that important reductions in surface spore contamination may be achieved during storage of the components prior to terminal sterilization. If circumstances permit storage at 60°C, even greater advantages may accrue.

In all of the foregoing experiments, it should be pointed out that D-value calculations have been used primarily for convenience in reporting the results. Observation of actual survival curves (see Figures 1.2-1.4), indicates that logarithmic count reduction plotted against time does not necessarily follow a straight line. Die-off appears to accelerate after a certain time period, particularly at high humidities, thus making the D-value estimates conservatively higher than they might otherwise be. Further investigation of the shape of the survival curves will be carried out.

The results of experiment 4, which measured the survival of natural spores from a soil sample, are illustrated in Figure 1.6 (see page 8) and are summarized in Table 1.3.

Table 1.3 - Survival of Spores from Natural Soil at 45°C (12-week Experiment, $N_o = 1.1 \times 10^3$)

Relative Humidity*	D-value (Days)
< 2%	127.0
50-56%	42.4
88-92%	19.5

* RH at 45°C

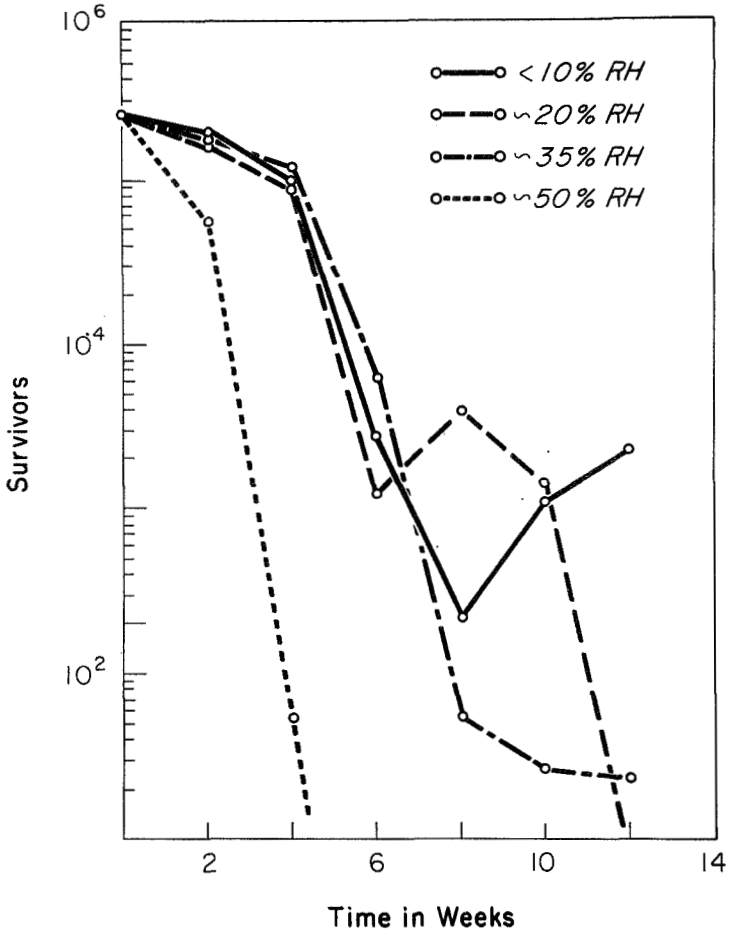

Figure 1.3 - Survival of Bacillus subtilis var. niger spores at 45°C at intermediate relative humidities.

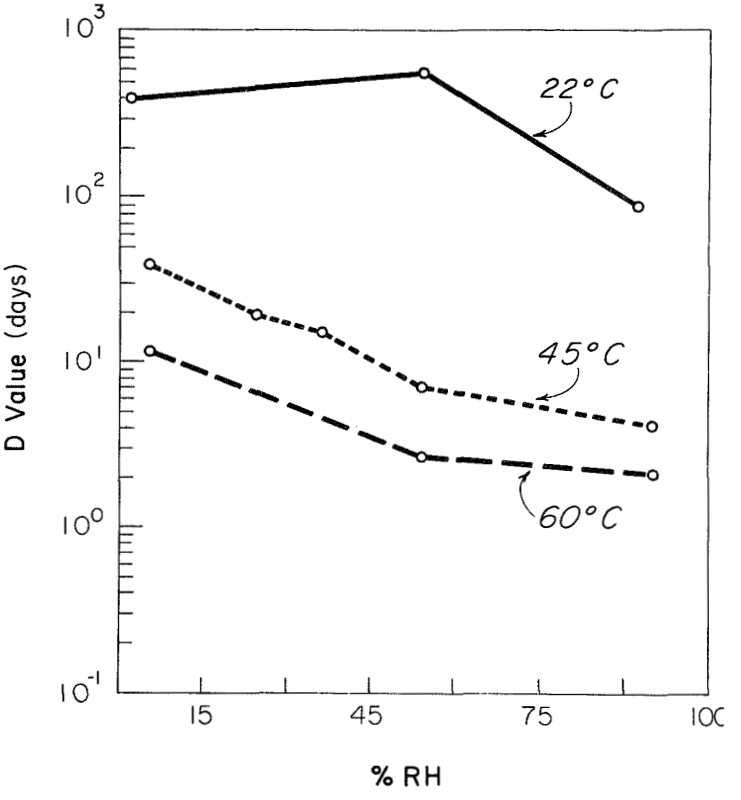

Figure 1.5 - The relationship of the D-value in day with relative humidity in percent for the data at 22, 45 and 60°C.

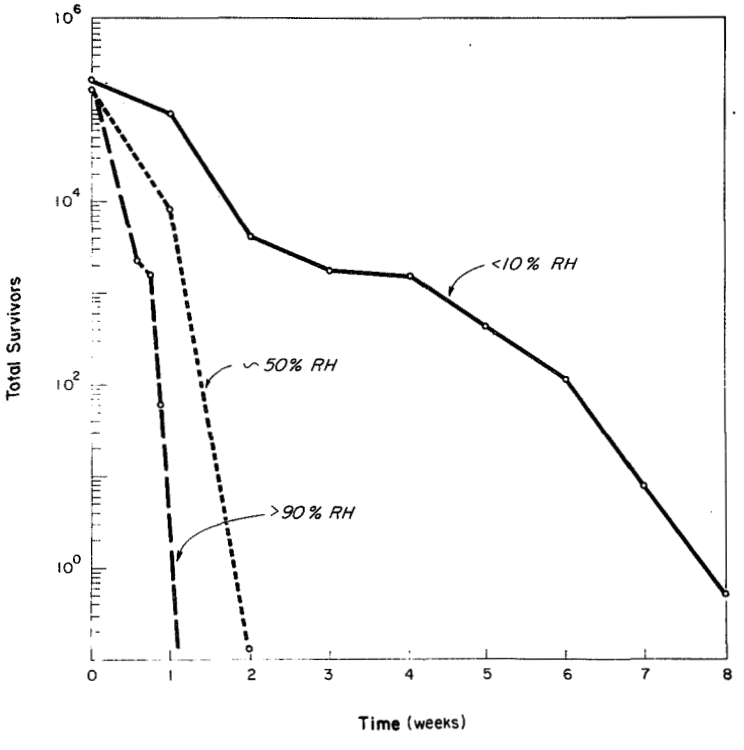

Figure 1.4 - Survival of Bacillus subtilis var. niger spores at 60°C as a function of relative humidity.

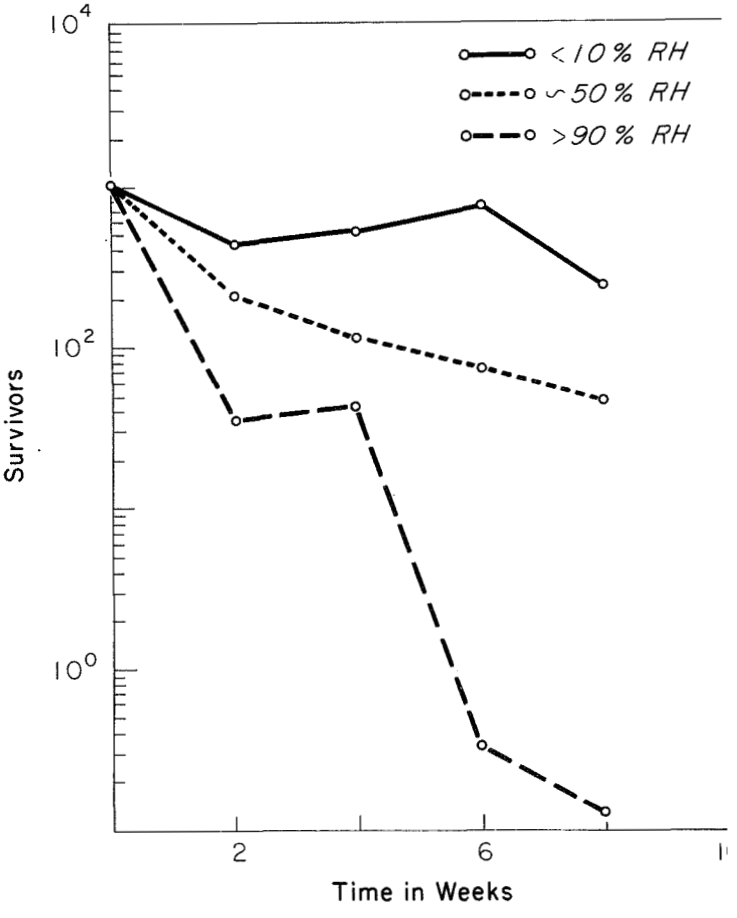

Figure 1.6 - Survival studies of spores obtained fro soil held for different periods of time at 45°C at several relative humidity levels.

It can be seen that the humidity effect at 45°C was similar to that observed for Bacillus subtilis var. niger spores. However, D-values for these spores are considerably longer. The results are not directly comparable, however, as N_o in this case was several logs lower than in other experiments.

Table I.4 summarizes the results to date of the series of experiments at 45°C.

Table I.4 - Survival of Bacillus subtilis var. niger Spores at 45°C

% RH	Strip Material	Colonies Surviving After: (weeks)						
		0	1	2	3	4	5	6
0.26-0.40*	SS	2.20E5	-	9.84E4	-	9.42E4	-	6.48E4
<2%	SS	3.09E5	-	1.45E5	-	1.41E5	-	6.22E4
	Glass	2.74E5	-	1.72E5	-	1.10E5	-	7.59E4
27-32	SS	3.09E5	-	2.23E5	-	1.10E5	-	1.74E4
49-55	SS	3.09E5	2.31E5	8.68E4	3.49E3	7.17E2	1.71E2	**
	Glass	2.74E5	8.38E2	**	-	-	-	-
90-95	SS	3.09E5	1.33E5	2.84E4	2.25E2	2.5E-1	7.5E-1	**
	Glass	2.74E5	8.90E1	**	-	-	-	-

* Humidity calculated at 45°C but measured during preparation only (at 23°C) -- not during storage.

** No colonies observed.

The spores used in this series are the same as those previously used. Different spore suspensions will be evaluated later. The results through six weeks are reported. There is no indication as yet, of a difference in survival rate between those spores in the less than 2% RH silica gel boxes and those prepared in the special dry gas environment at <1% RH. The very rapid die-away of the spores which were deposited on glass strips and stored at ∿50% or >90% RH was surprising. These spores died away so quickly that additional experiments will have to be performed in order to obtain sufficient data points to calculate the

D-values.

A secondary objective of this experiment has been to compare the data from replicate boxes, which each contained four strips, under identical conditions for each relative humidity. Data analyses from this objective will be presented in the next report.

Data from the experiment to determine the effect of agar concentration changes on the number of microorganisms recovered are summarized in Table 1.5.

Table 1.5 - Effect of Agar Concentration on Colony Counts

Quantity of Diluent Plated (a)	Agar Strength (b)	Quantity of Agar Added to Plate(ml) (c)	Relative Agar Strength in Pour Plate (d)*	Colonies per Strip times E-5	Mean Colonies per Strip times E-5
0.1	1 x	20	20/20.1	1.74 1.78 2.27 2.16 0.97	1.78
1.0	1 x	20	20/21	1.42 1.51 2.18 1.94 0.73	1.57
10.0	1 1/2 x	15	22.5/25	1.31 1.38 1.81 1.65 0.74	1.38
30.0	2 x	30	60/60	1.44 1.46 1.83 1.64 0.78	1.43

$$* (d) = \frac{(c) \times (b)}{(c) + (a)}$$

It appears that the original 0.1 ml from the 50 ml of diluent yielded somewhat higher counts than the other dilutions, however, no significant

differences were observed among the other three dilutions. This indicates that the variation in agar concentration generally achieved the goal of equalizing relative agar strength in the pour plates.

The results of experiment 7 are summarized in Table 1.6.

Table 1.6 - Comparison of Two Eppendorf Pipettes in Delivering 0.02 ml of Spore Suspension

	Pipette A		Pipette B	
	Colonies per Spore Deposit	Coefficient of Variation	Colonies per Spore Deposit	Coefficient of Variation
Trial 1 (8 deposits)	2.10E5 2.10E5	.11	2.37E5	.12
Trial 2 (8 deposits)	2.12E5	.17	2.15E5	.13
Mean of Two Trials	2.11E5	.14	2.26E5	.12

An analysis indicated that there was no significant difference which could be attributed to the pipettes even at $\alpha = 0.10$. Results also disclosed that there was no trend toward increased or decreased concentration of the spore deposit over the eight deposits made between the mixing sequences of the suspension.

CONCLUSIONS

At this time it is apparent that significant die-away of Bacillus subtilis var. niger spores does occur at relatively low temperatures (45°-60°C). This die-away is most pronounced at very high relative humidity (> 90%) but is also significant at intermediate humidities (∿ 50% or even lower). The same effect was noted for natural spores but with considerably longer D-values. Glass strips, when compared to stainless steel, appear to greatly accelerate the die-away at >50% RH, but the significance of this finding must await further exploration.

FUTURE WORK

In the immediate future experiments will continue to evaluate differences between glass and stainless steel, and the effect of different spore suspension histories. Later, priority will be given to experiments at higher temperatures (up to 90°C), using different surface materials and other spore species.